

# A novel 5' displacement spin-labeling technique for electron paramagnetic resonance spectroscopy of RNA

J.C. MACOSKO,<sup>1</sup> M.S. PIO,<sup>1</sup> I. TINOCO, JR., and Y.-K. SHIN

Department of Chemistry, University of California, Berkeley, and Structural Biology Department, Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720-1460, USA

## ABSTRACT

An RNA spin-labeling technique was developed using the well-characterized interaction between the HIV Rev peptide and the Rev response element (RRE) RNA as a model system. Spin-labeled RNA molecules were prepared by incorporating guanosine monophosphorothioate (GMPS) at the 5' end using T7 RNA polymerase and then covalently attaching a thiol-specific nitroxide spin label. Three different constructs of the RRE RNA were made by strategically displacing the 5' end within the native three-dimensional structure. Nitroxide-to-nitroxide distance measurements were made between the specifically bound RNA and peptide using electron paramagnetic resonance (EPR) spectroscopy. The dipolar EPR method can reliably measure distances up to 25 Å, the calculation of which is derived from the  $1/r^3$  dependence of the broadening of EPR lines in motionally frozen samples. This RNA-labeling technique, dubbed 5' displacement spin labeling, extends the usefulness of the dipolar EPR method developed for analysis of protein structure. The advantage of this technique is that it is applicable to large RNA systems such as the ribosome, which are difficult to study by other structural methods.

**Keywords:** circular permutation; dipolar method; GMPS; long range distance; nucleic acids

## INTRODUCTION

Although progress has been made in the past decade in understanding the various functions of RNA, biologically important nucleic acid molecules are still more difficult to characterize than proteins. Several well-established biophysical techniques have been recruited in an effort to better understand the biological role of RNA (Holbrook & Kim, 1998; Chang & Varani, 1997). A newly established method to quantitate distances by electron paramagnetic resonance (EPR) of spin-labeled molecules has been useful in probing protein structure and function, but has yet to be applied to RNA molecules. Given the utility of EPR in resolving problems in the structural biology of proteins (Hubbell et al., 1998; Shin, 1998), it would appear highly desirable to extend EPR as a tool for studying RNA.

Various aspects of spin-labeling EPR make it ideal for both protein and nucleic acid research. Spin labeling involves the site-specific attachment of a nitroxide group containing an unpaired electron to a macromol-

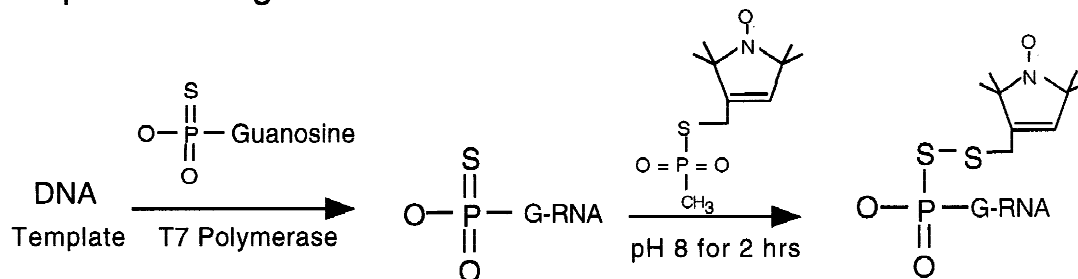
ecule. Consequently, the rotational correlation time of the nitroxide probe, which can be obtained from the EPR signal, yields information on macromolecular dynamics proximal to the site of attachment. The EPR method can be applied to very large molecules, including RNA-protein complexes such as the ribosome. Most importantly, EPR provides long-range distance measurements between two labeling sites up to 25 Å apart (Rabenstein & Shin, 1995). Furthermore, distance changes can be followed by EPR on the submillisecond time scale, which can provide information about the time course of conformational changes (Thorgeirsson et al., 1997).

In this article, we report on the development of a spin-labeling technique that illustrates the powerful advantages of EPR spectroscopy for the field of RNA research. The model RNA system we employ is the Rev response element (RRE), a secondary structural element found at the 5' end of HIV RNA that interacts with the HIV Rev protein to signal the transport of unspliced RNA out of the nucleus (Emerman & Malim, 1998). Our spin-labeling strategy (Fig. 1) has two components: (1) incorporating a guanosine monophosphorothioate (GMPS) at the 5' end of RNA and then spin labeling at the sulfur, and (2) designing functional RNA

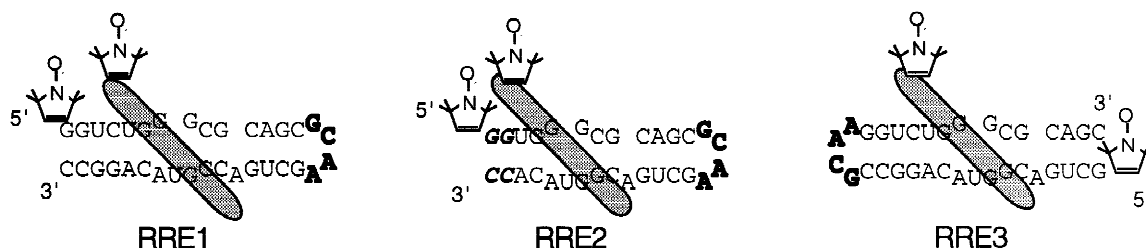
Reprint requests to: Y.-K. Shin, Department of Chemistry, University of California at Berkeley, Berkeley, California 94720, USA; e-mail: shin@cchem.berkeley.edu.

<sup>1</sup> Both authors contributed equally to this article.

## RNA Spin Labeling



## 5' End Displacement



**FIGURE 1.** A schematic of the RNA spin-labeling strategy. RNA is transcribed by T7 RNA polymerase in the presence of guanosine 5' phosphorothioate then spin labeled with S-(1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate spin label at pH 8 for 2 h. The sequence and secondary structure of the three RRE circular permutations are shown with spin labels attached to their 5' ends. The tetraloop, which moves in circular permutation, is indicated with bold nucleotides, and the two base pairs that move in 5' stem-length modulation are shown in bold italics. Diagonal gray rods represent the bound Rev with attached spin labels.

constructs with different 5'-end locations within the same three-dimensional structure. Three RRE constructs were designed for this study and used for determination of RNA-peptide distances by EPR. The results demonstrate that EPR is a useful technique in the study of RNA.

## RESULTS

The primary goal of this experiment was to develop a strategy for analyzing the structures and interactions of RNA using EPR techniques. Two major aspects of the project were (1) synthesizing spin-labeled RNA as well as the spin-labeled peptide and (2) measuring distances between the spin-labeled peptide and the spin-labeled RNA by EPR.

### Synthesis of spin-labeled RNA and peptide

We chose a small RNA-peptide system over an all-RNA system because RNA-protein interactions play a vital role in many biologically interesting systems. Furthermore, the presence of the peptide spin label enables us to attach only one label at a time to the RNA component, while still maintaining a doubly labeled system. For this study, we direct our efforts into developing a 5'-end RNA spin-labeling technique.

Other considerations for choosing a model system included the size of the RNA and the quality of available structural information. For the sake of simplicity while demonstrating this new method, the RNA-peptide system needed to be relatively small. Also, it was essential that the structure of the complex be known by an established high-resolution technique. By comparing this known structure with the distances obtained by EPR analysis, we could gauge the accuracy of our technique.

The HIV RRE-Rev system, characterized by Malim et al. (1989), fit these criteria well. RRE, the 11-kDa RNA component, has 30 bases of the 234-nt RNA structural element (G41-C54 and G64-79) connected by a GCAA tetraloop. The 3-kDa Rev peptide contains 17 amino acid residues (amino acids 32-52) from the arginine-rich region of the original 18-kDa Rev protein with an additional four alanines and one arginine at the C-terminus (Tan et al., 1993) and an aspartic acid at the N-terminus to increase helicity (Battiste et al., 1995). The overall molecular weight of the complex was 14 kDa, small enough for a test-case system. Additionally the solution state nuclear magnetic resonance (NMR) structure was solved by Battiste et al. (1996). This structure served as a standard for assessing the quality of our 5' spin-labeling technique.

The general strategy for spin labeling RNA is outlined in Figure 1A. GMPS was synthesized and included in the *in vitro* transcription reaction used for the synthesis of each RRE construct. Then the sulfur of the 5' GMPS was labeled with a thiol-specific nitroxide spin label. The details of this procedure are reported in Materials and Methods.

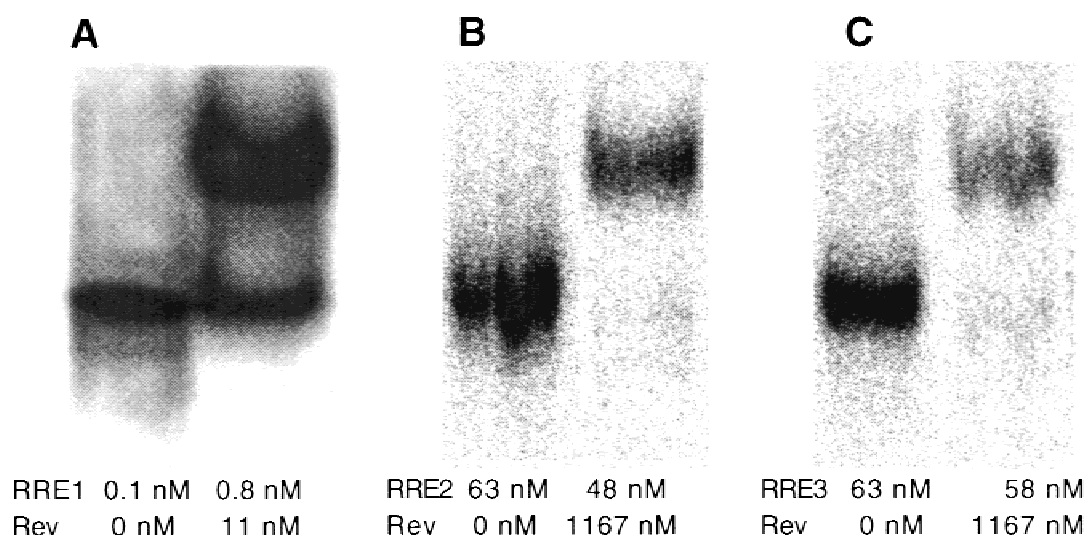
The three RRE constructs we chose to demonstrate the spin-labeling technique are shown in Figure 1B. With these constructs, we were able to place the 5' end, and thus a spin label, at several sites within the overall three-dimensional RNA structure. Construct RRE1, based on consensus sequence comparisons (Bartel et al., 1991), is the RNA sequence in the Rev-RRE complex whose structure was determined by NMR (Battiste et al., 1996). The RRE2 construct was made by deleting two base pairs from the stem region of RRE1, thereby moving the 5' end of RRE relative to the bound Rev peptide. This stem-length modulation tactic was used by Noller and coworkers in their study of tRNA analogs in the ribosome as an effective method of moving the 5' end without perturbing the global RNA architecture (Joseph et al., 1997). The RRE3 construct was made by a circular permutation of the RRE1 sequence. Circular permutation is best understood by thinking of the RNA sequence as a circle and then cutting the circle in different locations. This effectively displaces the 5' end but need not change the native secondary and tertiary structures. For example, Pace and coworkers (Harris et al., 1997) have used this approach to attach photoaffinity cross-linking groups to tRNA. By reversing the location of the tetraloop, we were able to move the site for spin labeling over 40 Å.

We classify these complementary techniques of stem-length modulation and circular permutation under the title of 5' displacement spin labeling. In either case the effect is the same: the 5' end is displaced to a specified location within the RNA three-dimensional structure. Once the 5' end has been positioned at a given site, GMPS can be added to the transcription reaction, thus providing a lone sulfur at the 5' end to react specifically with a thiol-containing spin label.

The procedure for spin labeling the Rev peptide is described in Materials and Methods. Alanine 51 was chosen as the best site for cysteine mutation and subsequent spin labeling, because of its proximity to the 5' end of the RRE1 construct and the absence of any base or backbone interactions to this region (Battiste et al., 1996). In fact, NMR results suggest that there is a wide pocket between Rev, position 51, and the adjoining region of RRE opening towards the C-terminal end of the peptide (see Discussion).

Once we obtained spin-labeled RRE and Rev peptide, we needed to examine the binding of the Rev peptide to the spin-labeled RRE variants qualitatively. Two assays were performed to determine this binding. First, we qualitatively measured the binding with a filtration experiment. Using a 10,000-molecular-weight cut-off Microcon membrane (Amicon, Massachusetts), RRE was concentrated 10-fold. Rev peptide not in the presence of RRE freely passed through the filter. However when Rev was added to RRE, Rev did not pass through the filter.

Second, gel shift assays (Fig. 2) were used to quantify the binding of each of the three spin-labeled RRE constructs to spin-labeled Rev. Excess spin-labeled Rev



**FIGURE 2.** Examples of RRE-Rev gel shift assays showing the change in mobility of radioactive RRE variants when Rev peptide is added. Binding reactions were carried out in the presence of excess tRNA at 4°C in a 10% polyacrylamide non-denaturing gel. **A:** Spin-labeled RRE1 at 0.07 and 0.8 nM bound with 0- and 11-nM spin-labeled Rev, respectively. **B:** Spin-labeled RRE2 at 53 and 48 nM bound with 0- and 1,167-nM spin-labeled Rev, respectively. **C:** Spin-labeled RRE3 at 63 and 58 nM bound with 0- and 1,167-nM spin-labeled Rev, respectively.

were added in varying concentrations to the spin-labeled RRE, which had been transcribed in the presence of  $^{32}\text{P}$  CTP and run on a native gel. The relative intensities of the two bands (protein-bound and free RNA) were integrated to obtain a semiquantitative binding constant for each construct. The results from all the gel shift assays performed for the three constructs at more than six different concentrations ranging from 0 nM to 1  $\mu\text{M}$  Rev peptide demonstrate that the spin-labeled Rev binds specifically to the spin-labeled RRE constructs with a dissociation constant ( $K_d$ ) approximately threefold greater than that observed for the binding of wild-type Rev peptide and RRE. Examples of gel shift experiments are shown for RRE1, RRE2, and RRE3 (Figs. 2A, 2B, and 2C, respectively).

### EPR spectroscopy

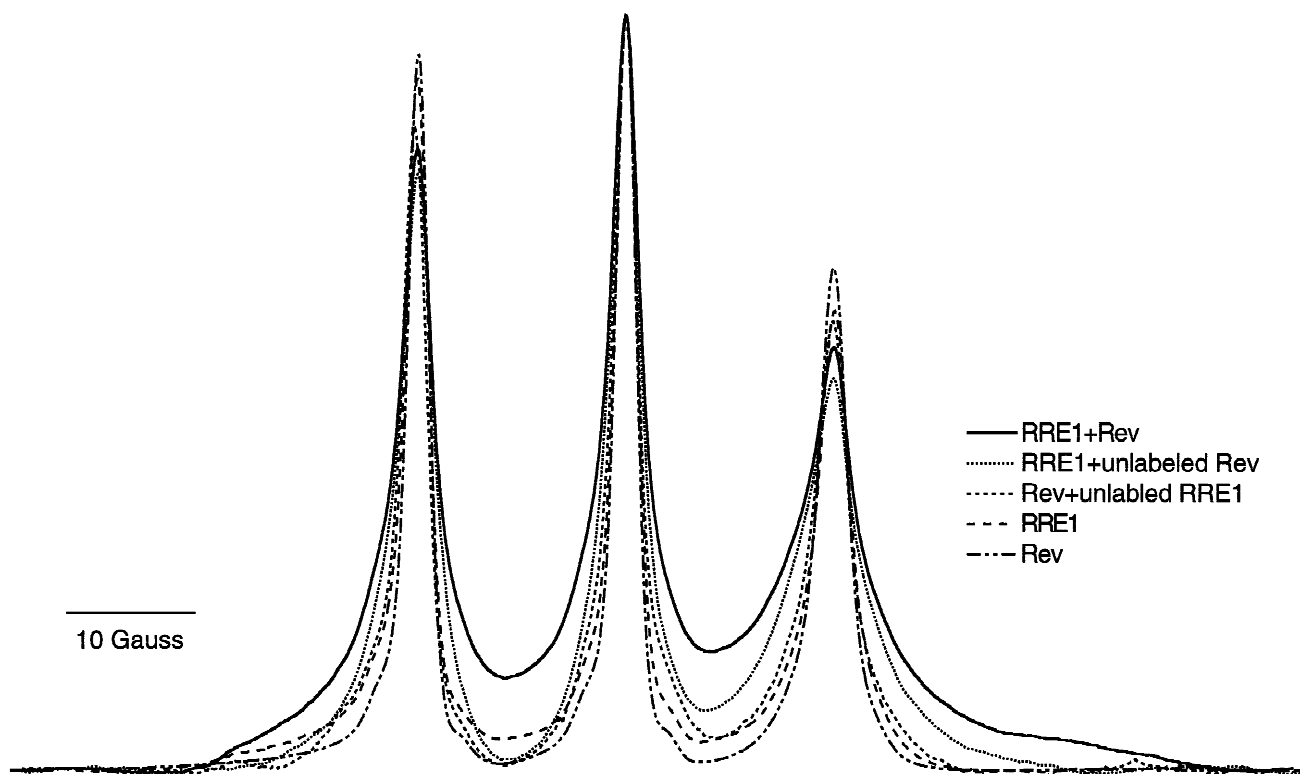
The spectra of unbound, labeled RRE and Rev (shown as a broken line and a hatched line, respectively, in Fig. 3), indicate that the synthesis of the labeled molecules was successful. These spectra show three relatively sharp absorption peaks, characteristic of a nitroxide covalently attached to a flexible chain. The covalent linkage of the spin label to the GMPS moiety was demonstrated by the fact that the integrated ab-

sorption did not change when samples were dialyzed for several days, whereas dialyzing after adding dithiothreitol eliminated the EPR signal.

The gel shift assays indicated that spin-labeled RRE and Rev form a specific complex, so we expected to see a broadening of the spectra due to the motional constraints placed on the spin label when Rev was added to the RRE constructs. Indeed, broadening upon complex formation is clearly visible in Figure 3 as an increased broadening for RRE plus unlabeled Rev (dotted line). Additional broadening is seen in RRE plus labeled Rev (solid line) due to dipolar interactions, which we examined in detail at low temperatures (see below). The broadening due to complex formation was also observed for RRE2 and RRE3 (data not shown); however, the additional broadening due to dipolar interactions was not seen in RRE3, consistent with the separation ( $>25 \text{ \AA}$ ) of the two probes predicted by the NMR structure (Rabenstein & Shin, 1995).

The relative spectral broadening diminished when Rev was added in more than, or less than, the optimal ratio of 1:1 (data not shown). The peaks became less broad due to the presence of free Rev or RRE.

Ultimately, we are interested in using this new technique to find distances within complexed RNA structures and obtain information about RNA function. To



**FIGURE 3.** Room-temperature EPR absorption spectra of bound and unbound Rev and RRE1. All spectra were taken at 22 °C in a Bruker ESP300 EPR spectrometer with a modulation amplitude of 0.5 G. The broadening in the bound and singly labeled systems is due to decreased nitroxide mobility. Additional broadening in the bound and doubly labeled system is due to spin-spin dipolar interactions.

measure distances between the spin labels in the RRE-Rev complexes, EPR spectra were collected at  $-140^{\circ}\text{C}$ . These absorption spectra are shown overlapped in Figure 4. The spectrum for RRE2-Rev is the broadest, followed closely by RRE1-Rev and RRE3-Rev. At low temperatures at which the molecular motion is completely frozen, the broadening of EPR spectra is exclusively due to dipole–dipole interactions between the two spin labels (Rabenstein & Shin, 1995). For labels that are proximal and more interacting, spectra are broader. Conversely, distant spin labels yield narrower peaks.

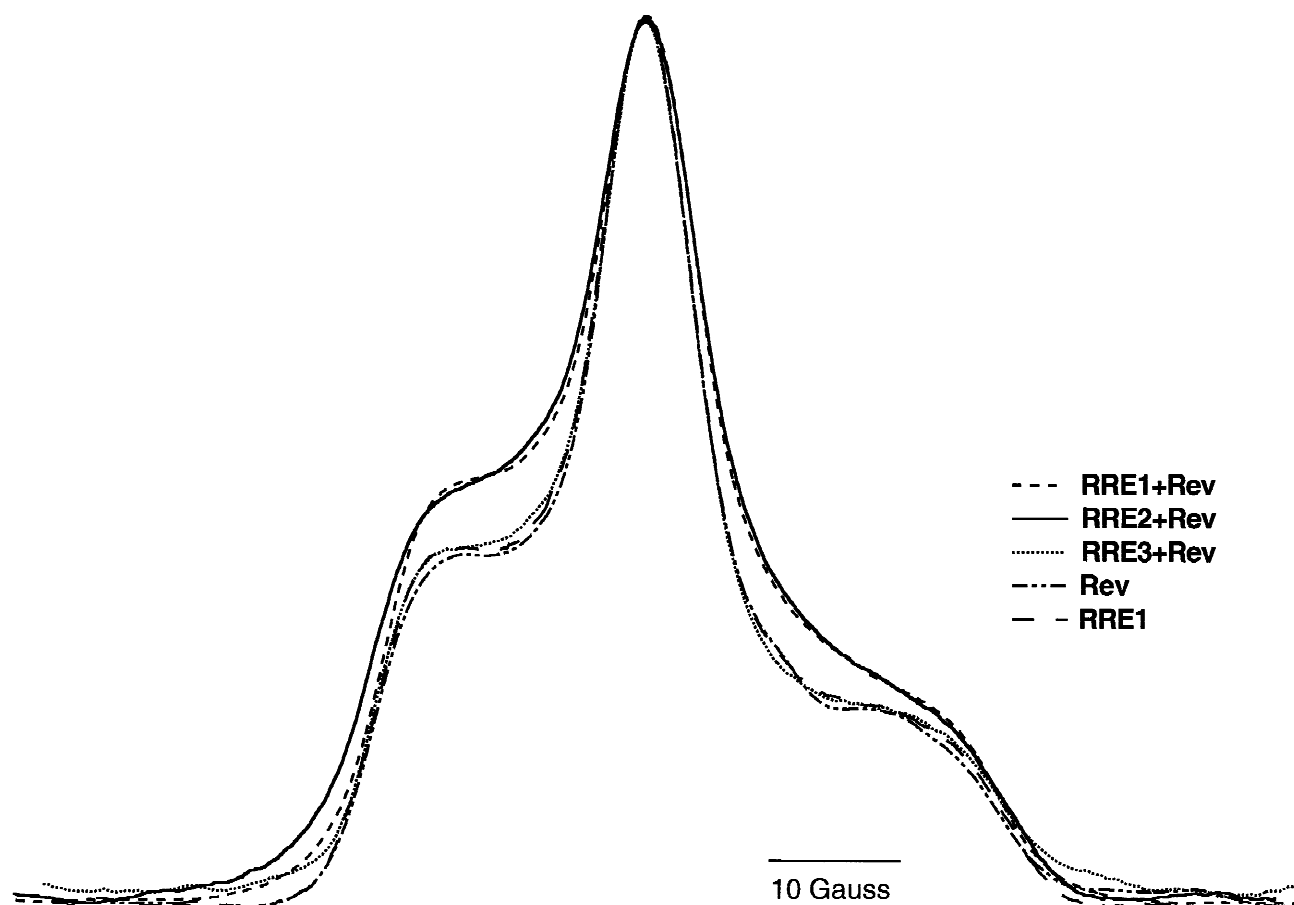
Qualitatively, then, it is clear that the distance between labels in the RRE3-Rev complex is greater than that in either the RRE1 or RRE2 complexes. Quantitatively, the overall spectral broadening, due to the splitting of EPR lines resulting from spin dipolar interactions, depends on the inverse cube of the distance ( $r$ ) between the spins. This is given by the equation

$$2B = 1.5g_e\beta(3\cos^3\theta - 1)/r^3$$

where  $2B$  is the splitting due to the spin–spin interactions,  $g_e$  is the isotropic  $g$  value of an electron,  $\beta$  is the electron Bohr magneton, and  $\theta$  is the angle between the interspin vector and the external magnetic field, which, for a frozen molecule, is fixed in time and is isotropically distributed. The magnitude of  $B$  is obtained from a Fourier analysis of a frozen EPR spectrum, a method developed by Rabenstein and Shin (1995).

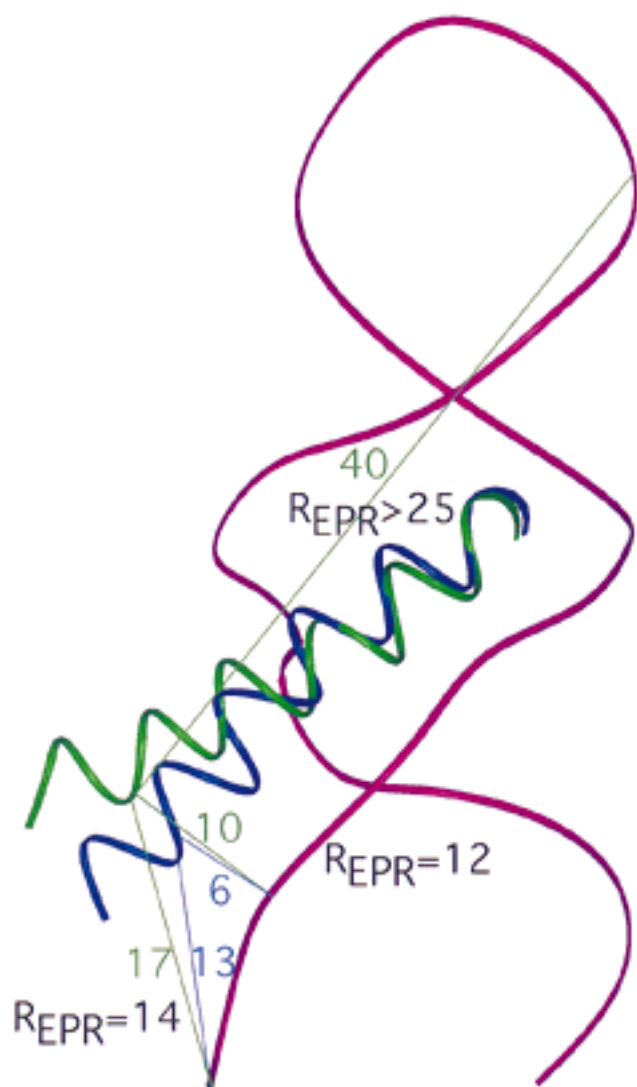
The resulting distances are given in Figure 5 in black with the modeled distances from two lowest-energy NMR shown in blue and green. The difference between the numbers in blue and green (Fig. 5) gives an estimate of the range of distances as determined by NMR.

For RRE1 the nitroxide tip-to-tip distance from the 5' end to Rev position 51 is  $14\text{ \AA}$ , whereas the modeled NMR distance,  $C_{\alpha}$ -P, is  $15\text{ \AA}$ . The RRE3-Rev distance, on the other hand, is modeled to be over  $40\text{ \AA}$  based on NMR data, which is well out of the  $25\text{-}\text{\AA}$  range of EPR (Rabenstein & Shin, 1995; Battiste et al., 1996). Thus, the EPR lineshape of RRE3-Rev at low temperatures (Fig. 4) is indistinguishable from a singly labeled com-



**FIGURE 4.** Low-temperature EPR absorption spectra of bound and unbound Rev and RRE constructs. The spectra were collected in a quartz vacuum dewer held at  $-140^{\circ}\text{C}$  with the modulation amplitude set to  $3.2\text{ G}$ . The broadening of the RRE1 and RRE2 spectra indicates spin–spin interactions.





**FIGURE 5.** The average NMR structure of RRE (violet, Protein Data Bank (PDB) file 1ETF) complexed with two out of the 19 lowest-energy Rev structures (blue and green, PDB file 1ETG), which show the range of Rev-RRE distances. EPR-measured nitroxide tip-to-tip distances are shown in black, and the corresponding  $C_{\alpha}$ -P distances of the two different NMR structures are shown in green and blue. The green helix is a structure chosen to represent the closest NMR modeled distances from the 19 lowest-energy conformations, whereas the blue helix represents the longest modeled Rev-RRE distance. Together, these helices give an estimate of the upper and lower limit in the range of possible  $C_{\alpha}$ -P distances based on NMR data. For EPR nitroxide tip-to-tip distances, the main source of error is due to the flexibility of the nitroxide-bearing side chains (Rabenstein & Shin, 1995). A simple molecular-dynamics calculation indicated that this error is in the range of 2 to 2.5 Å (Fiori & Millhauser, 1995). Errors resulting from EPR spectral measurement are negligible.

plexed spectrum. In the case of RRE2-Rev, the measured tip-to-tip distance is 12 Å and the modeled  $C_{\alpha}$ -P based on NOE constraints is between 6 and 10 Å.

## DISCUSSION

The purpose of this experiment was to extend the powerful spin-labeling EPR technique to the field of RNA

research. First, a suitable model system was chosen and a strategy for sitespecifically labeling RNA was developed. Second, using previously calibrated EPR dipolar methods, distances were determined and compared with NMR results. Both goals were accomplished in this study and are discussed below followed by an assessment of the usefulness of this new technique.

## Current spin-labeling systems and strategies

For site-specific spin labeling of proteins, the residue of interest is mutated to a cysteine, then labeled with thiol-specific nitroxide spin labels (Altenbach et al., 1990). The native cysteine residues are typically mutated to alanine to avoid unwanted spin labeling (Hwang et al., 1999). The main obstacle to site specifically labeling RNA is that within RNA there are no naturally occurring attachment sites analogous to the sulfur of cysteine. One possibility would be to chemically synthesize the RNA to include a spin label, as has recently been explored by Ramos and Varani (1998). This method becomes more difficult as the length of the RNA is increased. One of the goals of this experiment was to provide a technique that can be applied to RNA molecules of any length. We therefore opted for the strategy of cyclic permutation of the 5' end as outlined in Figure 1.

## Distance determination

Since the publication of the "EPR spectroscopic ruler" for the determination of distances in protein by Rabenstein and Shin (1995), this method has been applied successfully to many problems involving proteins (Rabenstein & Shin, 1996; Hall et al., 1997; Thorgeirsson et al., 1997; Ottemann et al., 1998). Now, we extend the same spin-labeling EPR strategy to RNA or RNA-protein systems. For RRE-Rev model systems, we applied the ruler to three categories of 5'-displacement spin-labeling constructs. These constructs (RRE1, RRE2, and RRE3), representing native, permuted, and modulated 5' ends, form a reasonable test of the method and yield three unique distance measurements. The distances measured for the RRE constructs correlate well with the NMR-based structure. Specifically, the EPR-determined distance for RRE1-Rev is slightly shorter relative to the average NMR-based structure, whereas the RRE3-Rev distance is relatively longer. This could be explained by the methanethiosulfonate spin label (MTSSL) moiety being positioned in such a way that the nitroxide group is pointing towards the C-terminus of Rev. If the position of the nitroxide was shifted along the 5-Å length of MTSSL, it would account for the observed EPR distances in RRE1 and RRE2. Also, the 19 low-energy structures calculated from NMR data show high flexibility in the C-terminal region of Rev. This higher flexibility could account for

the larger distance observed by EPR spectroscopy. However, some differences between the interpretation of the data in this model RNA study and in previous protein EPR studies must be examined. From extensive studies in proteins, it has been established that a large number of EPR distances compensate for the spatial uncertainty of the nitroxide groups in structural determination.

Distance determination is the primary prerequisite for structural studies. It is encouraging that the EPR method outlined in this study compares favorably with the previously determined NMR structure. More importantly, this distance technique is poised to study larger, more complicated RNA systems, whose size would prohibit NMR strategies from being effectively employed. The application of this technique in the future promises to be a fruitful area of RNA research.

### Future direction

An inherent limitation of GMPS incorporation could be that the site of attachment is at the 5' end. This means the circular permutations and 5' displacement constructs must be designed so as not to perturb the function. An RNA-dependent RNA polymerase that incorporates RNA primers during transcription has been recently reported (Kao et al., 1999). This enzyme can be used to transcribe large intact RNA using a 10–30 nt RNA synthetic primer that contains a spin-label attachment site, or contains a nitroxide. This technique would allow us to position the spin label at any site within the first 10–30 nt. This work is well under way.

### Conclusion

In the past several years, spin-labeling EPR has proven to be a very useful technique in the study of protein structure and function. In 1998, the structure of the neuronal SNARE complex was determined based on EPR distance and was a powerful demonstration of the ability of EPR to be a structural technique (Poirier et al., 1998). In systems where other techniques have difficulty, such as with membrane proteins, EPR has proven itself to be extremely valuable (Altenbach et al., 1994; Macosko et al., 1997). Furthermore, the ability of EPR to resolve dynamic changes in proteins, sometimes in a time-resolved fashion, has been exploited to study the function of several important proteins.

As new X-ray crystal structures of RNA are determined, EPR and the different spin-labeling methods can be used to monitor the function of these RNA systems. For example, the structure of the ribosome will provide the structural background needed for a detailed understanding of translation. The 5'-displacement spin-labeling technique can be used to track the movement of tRNA, mRNA, and essential parts of the large and small subunit rRNA, while at the same time monitoring

the key ribosome-associated proteins. These studies could be done in a time-dependent manner to follow changes on a millisecond time-scale and thus greatly facilitate the elucidation of the translation mechanism.

By establishing the usefulness of spin-labeling EPR in an RNA–protein system, the possibilities for understanding the world of RNA are more numerous. There are many opportunities to expand the technique itself and extend its applicability to other systems. In the near future, this method may become an important tool for the study of RNA and the many roles it plays in all organisms.

### MATERIALS AND METHODS

The synthesis of the mutant Rev peptide was complicated by the presence of 11 arginine residues out of 23 total residues. Mass spectrometry showed several minor peaks indicating the absence of one, two, or more arginine residues. Coupling the arginine residues twice as long as the other residues reduced the number of side peaks. The Rev mutant peptide was synthesized on an Applied Biosystems 431 synthesizer using Rink amide MBHA resin (Nova Biochem) and fluorenylmethoxycarbonyl (Fmoc) protection. Coupling reactions lasted 2 h except for the arginine side chains, which were allowed to couple for 4 h. The peptides were cleaved and deprotected for 4 h in TFA-containing Reagent K (King et al., 1990), filtered, and deprotected again for 4 h. The volume of TFA was reduced by rotary evaporation and peptides were precipitated into an ice-cold tert-butyl methyl ether (tert-BME). After 5–10 washes of cold tert-BME, peptides were desiccated overnight. Reversed-phase high-performance liquid chromatography (HPLC) (Rainin) was done on a Vydac C-18 resin column using a gradient from 10–40% acetonitrile in 0.1% TFA. Samples were frozen and lyophilized, then analyzed by electrospray (Hewlett-Packard Model 5989A) mass spectrometry.

Following the procedures outlined earlier (Yu et al., 1994) the Rev peptide was spin labeled using a twofold excess of S-(1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl) MTSSL for 2 h in a 5-mM potassium phosphate buffer (pH 7.5) at room temperature, or overnight at 4 °C. Fully spin-labeled peptides were purified by C-18 HPLC and analyzed by EPR. Fractions of spin-labeled Rev were lyophilized and stored for later use. Typically, aqueous peptides degraded after two weeks at 4 °C.

Guanosine monophosphorothioate was synthesized following a protocol developed by Pace and coworkers (Harris et al., 1997). Guanosine was added to triethyl phosphate at 100 °C. The mixture was stirred overnight at 4 °C and barium acetate was added to precipitate the product. Triethylamine and ethanol were added and the precipitate pelleted. The pellets were solubilized by vortexing with Dowex50WX8 H+ exchange resin. GMPS was separated on a Supelco TSK-gel Toyopearl DEAE-650M column with a 0–0.4 M ammonium bicarbonate gradient. The purity was assessed by thin layer chromatography on a polyethylene imine plate in 1 M lithium chloride. Further analysis was done by UV spectroscopy yielding an extinction coefficient of 12,100 and a 280-nm:260-nm ratio of 0.62. Samples were rotovapped, washed once with 80% ethanol, twice with 80% ethanol containing 1.4% triethylamine, and once again with 80% ethanol.

Proton NMR spectra of the purified GMPS matched within  $\pm 0.05$  ppm of the published GMP NMR spectra (Aldrich). A small peak corresponding to guanosine (Aldrich) was also observed. The integrated areas of the peaks showed that less than 5% unreacted guanosine was present in the purified GMPS. No other significant impurities were detected by  $^1\text{H}$  NMR.

Three constructs of RRE (Fig. 1) were synthesized by T7 in vitro transcription. Transcription buffer, primer, and template DNA, ATP, CTP, and UTP were added according to previous protocol (Milligan et al., 1987; Wyatt et al., 1991). GTP was added at 76% of normal concentration and GMPS was added at three times the concentration of GTP. After transcription, RNA was ethanol precipitated overnight and loaded on a 20% polyacrylamide denaturing gel. Typically a single broad band was visible by UV shadowing, although for the RRE2 construct, a lower band was visible, presumably due to 5' end GTP incorporation, as this RNA could not be spin labeled. Deoxyguanosine-5'-phosphorothiolate (dGMPS; USB, Ohio) was used in an attempt to improve the sulfur incorporation at the RRE2 5' end, but dGMPS proved no more effective than GMPS. RNA was electroeluted and ethanol precipitated. The RNA was resuspended in pH 8 buffer and labeled with MTSSL for 2 h at room temperature. Samples were dialyzed three days against the final buffer (10 mM HEPES-KOH, pH 7.5, 100 mM KCl, 1 mM  $\text{MgCl}_2$ ).

The full protocol for the RRE-Rev gel shift assay was kindly provided by Dr. Damian McColl in Professor Alan Frankel's laboratory at the University of California, San Francisco. Analytical amounts of the three RRE constructs were transcribed in the presence of  $^{32}\text{P}$ -labeled CTP (Amersham) and GMPS. After a 3-h T7 transcription reaction, samples were loaded on a 20% polyacrylamide gel containing 7 M urea. Bands were cut out and soaked in 600 mM NaOAc buffer (pH 6.0) with 1 mM EDTA and 0.01% SDS at 4°C for 2 h. This elution buffer was extracted and diluted in 2.5 vol of EtOH and incubated on ice for 10 min. The precipitate was pelleted by centrifugation for 10 min at 14,000 rpm on an Eppendorf tabletop centrifuge, model 5415C.

EPR spectra were measured using a Bruker ESP300 EPR spectrometer (Bruker, Germany) equipped with a low-noise amplifier (Mitech) and a loop-gap resonator (Medical Advances). The modulation amplitude was set at 0.5 G for room-temperature spectra and 3.2 G for low-temperature spectra. The loop-gap resonator was inserted into a quartz vacuum dewar and the temperature was lowered using liquid nitrogen. Fourier deconvolution was performed on an indigo II SGI (Silicon Graphics, California) running Matlab (The Math Works Inc.). The details of the deconvolution procedure are reported elsewhere (Rabenstein & Shin 1995). Prior to low-temperature measurements, 10% glycerol was added to the samples to stabilize the complexes. UV spectroscopy measurements of RRE concentration, when compared with the EPR-integrated spin concentrations, confirmed that RRE was greater than 95% spin labeled.

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